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Purinergic receptors in the carotid body as a new drug target for controlling hypertension

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In view of the high proportion of individuals with resistance to antihypertensive medication and/or poor compliance or tolerance of this medication, new drugs to treat hypertension are urgently needed. Here we show that peripheral chemoreceptors generate aberrant signaling that contributes to high blood pressure in hypertension. We discovered that purinergic receptor P2X3 (*P2rx3*, also known as *P2x3*) mRNA expression is upregulated substantially in chemoreceptive petrosal sensory neurons in rats with hypertension. These neurons generate both tonic drive and hyperreflexia in hypertensive (but not normotensive) rats, and both phenomena are normalized by the blockade of P2X3 receptors. Antagonism of P2X3 receptors also reduces arterial pressure and basal sympathetic activity and normalizes carotid body hyperreflexia in conscious rats with hypertension; no effect was observed in rats without hypertension. We verified P2X3 receptor expression in human carotid bodies and observed hyperactivity of carotid bodies in individuals with hypertension. These data support the identification of the P2X3 receptor as a potential new target for the control of human hypertension.

One-third of the human population has hypertension. In the USA, despite the fact that 75% of individuals use antihypertensive medication, in only 53% (~41 million) of those taking such medication is blood pressure controlled[1]. This low rate of control might, in part, reflect a serious problem with drug adherence, owing to the asymptomatic nature of hypertension and the poorly tolerated side effects of current medications. Substantial savings in healthcare costs would result if an individual's blood pressure could be reduced to <140/90 mmHg[2]. In adults over 40 years of age, there is clear evidence that a 10-mmHg rise in diastolic blood pressure (DBP) above normal levels (80 mmHg) doubles the risk of death from cardiovascular disease[3]. Furthermore, at least 8% of an estimated 900 million people with hypertension worldwide[4] have developed and currently experience drug resistance[5,6]. Categorically, there remains an unmet clinical need for ways to control blood pressure.

The most recently introduced antihypertensive drug is aliskiren, a renin inhibitor released in 2007. This was the first new drug to emerge for hypertension in 13 years, yet its efficacy beyond the existing renin-angiotensin–aldosterone enzyme inhibitors and receptor blockers is questionable, and associated adverse effects call into question its long-term benefit–harm relationship[7]. The paucity of new pharmacological antihypertensive agents has led to a series of device-based interventional studies, including renal denervation[8], stimulation of carotid baroreceptors[9], deep brain stimulation[10] and arterial venous anastomosis[11,12]. Here we introduce a new mechanistic approach that uses a highly selective small-molecule antagonist, and we provide preclinical and clinical data to justify its use in a future clinical trial for hypertension.

Currently, the carotid body is under consideration as a therapeutic target for cardiovascular disease. The carotid body chemoreflex-evoked sympathoexcitatory response is potentiated in both a preclinical model of hypertension—the spontaneously hypertensive (SH) rat—and in humans with hypertension[13–18], and denervation of the carotid body is evidently an effective way to control both the development and maintenance of high blood pressure in the SH rat[19,20]. On the basis of these findings, we concluded that in conditions of hypertension, the carotid body chemoreceptors generate aberrant excitatory tone that drives up sympathetic activity and causes high blood pressure[14,21]. Indeed, we found that when this carotid body tone is switched off through hyperoxia or nerve section to disconnect the carotid body from the brain, arterial pressure and sympathetic activity are reduced in SH rats, but are unaffected in a normotensive rat strain[14]. In humans with hypertension, hyperoxia lowers sympathetic activity[18], and carotid body resection can reduce arterial pressure substantially[22]. Thus, these animal and human data support the presence of aberrant carotid body discharge as contributing to hypertension. Although the mechanistic basis for the carotid body aberrant tone is unknown, it could be pivotal for refining new pharmacological antihypertensive approaches, as we propose here.

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A plethora of mechanisms governs carotid body signaling[23–27]. In our current investigation, we have studied the role of adenosine triphosphate (ATP)-gated ion channels (called purinergic P2X receptors), specifically the C-fiber-localized, P2X3-receptor subtypes, which are commonly associated with afferent sensitization and might contribute to hyperreflexic disease states in a variety of organs[28,29]. ATP is one of a number of transmitters involved in hypoxic signaling in the carotid body[30–35]. P2X3-receptor expression is present in both carotid bodies and petrosal ganglia neurons of normotensive rats[36]. Moreover, combined P2X2- and P2X3-subunit deletion in mice reduces the ventilatory response to hypoxia, as compared to wild types[37]. We hypothesized that the P2X3 receptor contributes to both peripheral chemo-hyperreflexia and aberrant discharge of the carotid body in hypertension, and that blockade of this receptor would cause an antihypertensive effect. To address this, we have tested the effects of highly selective P2X3-receptor antagonists on hypertension in rats and have demonstrated the applicability of these preclinical findings to humans with hypertension.

RESULTS

Hyperactivity of carotid bodies in hypertensive rats

We first tested whether the carotid body was more active in conditions of high blood pressure. We recorded carotid body afferent discharge from the carotid sinus nerve of the *in situ* preparation (an unanesthetized decerebrated rat on cardiopulmonary bypass). Sodium cyanide (NaCN), simulating hypoxia, was used to stimulate the peripheral chemoreflex response because this treatment provides a specific, quantifiable and temporally controlled stimulus that is repeatable over a course of hours. Both basal discharge and chemoreflex-evoked volleys (15–30 µg NaCN, intra-arterially (i.a.)) were elevated in rats with hypertension, as compared to those without hypertension (SH versus Wistar rats: basal, 24.9 ± 2 versus 1.4 ± 0.3 spikes/s, respectively, $P < 0.001$; chemoreflex, 62.3 ± 3 versus 28.3 ± 2 spikes/s, respectively; $P < 0.001$, **Fig. 1a–c**). Next, we used a low dose of dopamine to inhibit carotid body discharge[38,39]. In SH rats *in vivo*, dopamine infusion (10 µg/kg/min intravenously (i.v.), as described[39]) depressed respiration, an effect not observed in Wistar rats (**Fig. 1d**), which suggests the presence of carotid body tonicity in animals with hypertension. During intracellular recordings in the *in situ* preparation, neurons were characterized as responding to the NaCN stimulus, characterizing them as chemoreceptive primary afferent petrosal ganglion neurons. Such neurons, recorded from hypertensive rats, were more depolarized (−51.5 ± 0.9 versus −55.7 ± 1 mV; $P < 0.001$, **Fig. 2a**,**c** and **Supplementary Fig. 1**), exhibited tonic firing (1.5 ± 0.8 versus 0 Hz; **Fig. 2a**) and displayed an enhanced chemoreflex-evoked firing response to an equivalent dose of NaCN (22.5 µg, 123 ± 5 versus 52 ± 3 spikes; $P < 0.001$, **Fig. 2a**,**d**), as compared to normotensive rats. In sum, when compared to the normotensive rat, the carotid body of an SH rat is hyperactive, generating both aberrant tone and hyperreflexia.

Chemoreceptive petrosal neuron excitability in SH rats

We next compared the electrical excitability of chemoreceptive petrosal neurons in SH and Wistar rats. We found that the neurons recorded from SH rats, as compared to those from Wistar rats, exhibited enhanced excitability to injected current pulses; for example, a 1 nA current pulse evoked 25 ± 1.1 spikes in SH rats versus 15 ± 1.7 spikes in Wistar rats (**Supplementary Fig. 2a**,**b**, $n = 6$; $P < 0.05$). Neither the membrane input resistance of these chemoreceptive petrosal neurons (119 ± 3 versus 117 ± 9 MΩ; **Supplementary Fig. 1c**) nor their whole-cell capacitance (**Supplementary Fig. 1d**) was different between the rat strains.

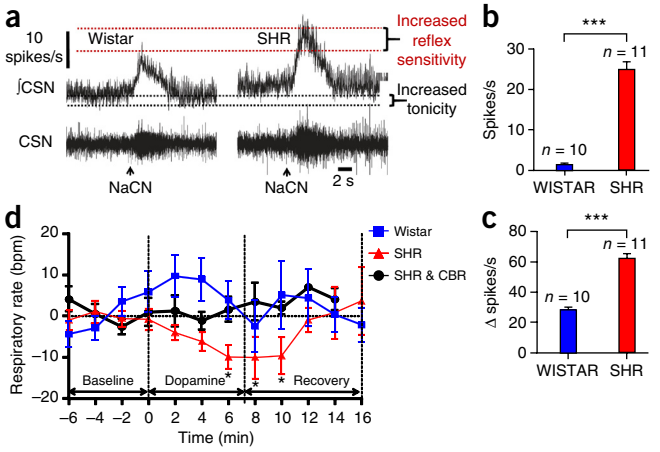

Figure 1 Overactive peripheral chemoreceptors in spontaneously hypertensive (SH) rats. (**a**) Original recordings of raw and integrated carotid sinus nerve (CSN and ∫CSN, respectively) activity from the *in situ* perfused preparation, showing both basal discharge and discharge evoked reflexively through stimulation of the carotid bodies with sodium cyanide (22.5 µg NaCN i.a., arrows) in SH rats (SHR) and Wistar rats. (**b**,**c**) Quantitation of the results from the experiment in **a**, showing spikes/s (**b**) and the change in the number of spikes/s after NaCN stimulation (**c**) (one-way ANOVA Bonferroni post test; $n = 10$ or 11; $^{***}P < 0.001$). (**d**) Respiration rate in conscious radiotelemetered Wistar and SH rats with or without selective carotid body resection (CBR) performed in separate groups of SH rats. Dopamine (10 µg/kg/min i.v.) was infused as indicated to silence endogenous carotid body activity. One-way ANOVA Dunnett's post test. $n = 5$ per group; $^{*}P < 0.05$. Data for each rat group are compared to the group's own baseline. All data are means ± s.e.m.

P2X3-receptor antagonism normalizes chemoreceptive petrosal neuron excitability in SH rats

Given that the P2X3 receptor is expressed in the carotid body of normotensive rats[36] and is associated with pathological afferent sensitization in numerous other organs[28,29,33], we assessed whether this receptor is involved in carotid body dysfunction in SH rats *in situ*. In SH rats, focal delivery of a highly selective, noncompetitive P2X3-receptor antagonist (AF-353; 15 nl, 20 µM[40]) via a glass micropipette inserted into the ipsilateral carotid body caused hyperpolarization (−9.9 ± 2.1 mV; $n = 12$, $P < 0.001$; **Fig. 2b**,**c**) and total abolition of ongoing activity (**Fig. 2b**), as well as a reduction in the chemoreflex sensitivity of petrosal chemoreceptive neurons (from 123 ± 5 to 34 ± 2 spikes; $n = 12$, $P < 0.001$, **Fig. 2b**,**d**). In normotensive animals *in situ*, AF-353 delivery also caused hyperpolarization (−4.4 ± 1.8 mV; $n = 10$, $P < 0.001$; **Fig. 2c**) and reduced the chemoreflex-evoked volley (from 52 ± 3 to 32 ± 2 spikes; $n = 10$, $P < 0.001$; **Fig. 2b**,**d**). The magnitudes of both the hyperpolarization and the reduction in chemoreflex-evoked spiking were greater in SH rats than in Wistar rats ($P < 0.001$; **Fig. 2c**,**d**). Notably, during P2X3-receptor antagonism, carotid body activation yielded comparable firing responses in petrosal neurons between the rat strains (SH, 34 versus Wistar, 32 spikes; not significant (n.s.)); this effect of P2X3 antagonism was reversible in SH rats upon washout of the drug (**Fig. 2b**). After AF-353 treatment, the intrinsic firing response to injected depolarizing current pulses was reduced in SH rat neurons (for example, at 1 nA, 25 ± 1 to 19 ± 1

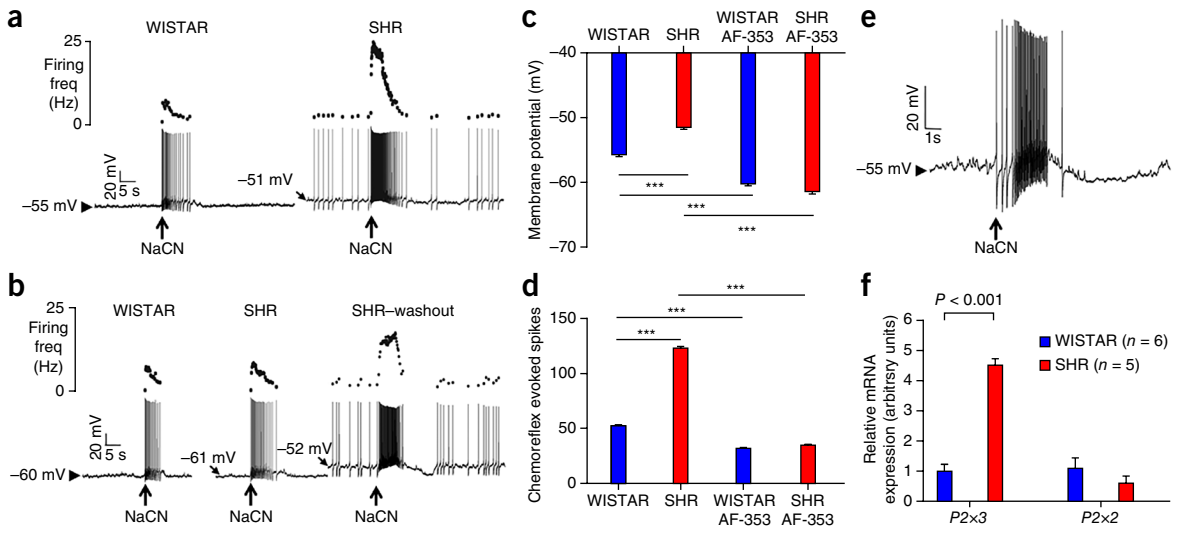

Figure 2 P2X3-receptor-mediated hyperreflexia and tonicity of chemoreceptive petrosal neurons in spontaneously hypertensive (SH) rats are associated with the upregulation of *P2x3* receptor mRNA. (**a**) Representative whole-cell patch clamp recordings from chemoreceptive petrosal neurons from a Wistar (left) and SH rat (SHR, right) recorded from the preparation *in situ*. Ongoing discharge, membrane potential and reflex-evoked responses to carotid body stimulation (NaCN, sodium cyanide, 22.5 µg i.a., arrows) were compared between rat strains. (**b**) Recordings of neurons as in **a** after P2X3-receptor blockade with AF-353 (20 µM, 20 nl), which was delivered focally into the ipsilateral carotid body by picoinjection. Also shown is a recording from the same neuron from an SH rat after AF-353 washout. (**c**,**d**) Grouped mean data quantifying membrane potential (**c**; see also **Supplementary Fig. 1a**,**b**) and chemoreflex-evoked firing responses (**d**) with and without P2X3-receptor antagonism in Wistar and SH rats. Data in (**c**) are means ± s.d., and those in (**d**) are means ± s.e.m. One-way ANOVA Bonferroni post-test (n = 12 SH, n = 10 Wistar rats). (**e**,**f**) Single-cell RT–qPCR for *P2x3* and *P2x2* was performed in petrosal chemoreceptive neurons in the *in situ* arterially perfused preparation. Given that the petrosal ganglion contains neurons with different sensory modalities, RT–qPCR was performed only on those cells defined as chemoreceptive, as assessed by their activation with a chemoreceptor stimulus (NaCN, 22.5 µg). Shown are a recording from one such chemoreceptive neuron (**e**) and quantitation of *P2x3* and *P2x2* mRNA (n = 5 or 6 neurons). Two-way ANOVA Bonferroni post-test. Data in **f** are means ± s.e.m. ***P < 0.001.

spikes; P < 0.05; **Supplementary Fig. 2a**,**b**), but not in Wistar rat neurons (15 ± 2 to 12 ± 2, n.s.).

P2X3 receptors are upregulated in petrosal neurons of SH rats

We next tested whether the expression of P2X3 receptors is upregulated in SH rat petrosal neurons. Single-cell PCR analysis of characterized chemoreceptive petrosal ganglion neurons recorded from SH rats *in situ* (**Fig. 2e**) revealed a greater than four-fold upregulation of *P2x3* receptor mRNA relative to that in Wistar rats (**Fig. 2f**; n = 6, P < 0.001). A comparable upregulation of the P2X3 receptor at the protein level in carotid bodies was confirmed by western blot (**Fig. 3a**). Notably, no difference in *P2x3* or *P2x2* mRNA expression in nonchemoreceptive petrosal neurons was observed between the rat strains (**Supplementary Fig. 2e**), which is indicative of specificity to chemoreflex neurons. Moreover, there were no differences between the rat strains in their expression of *P2x2* (**Fig. 2f**) or tyrosine hydroxylase (a marker of glomus cells) mRNA expression in either petrosal chemoreceptive neurons (**Supplementary Fig. 2c**) or in chemoreceptive neurons located in the nucleus tractus solitarii (**Supplementary Fig. 2f**). P2X3 receptors were observed in tyrosine-hydroxylase-positive glomus cells (**Fig. 3b**), as well as in axons within the carotid body (**Supplementary Fig. 3a**,**b**).

SH rat chemoreceptive petrosal cells are sensitized to ATP

On the basis of the upregulation of *P2X3* receptor expression in the chemoreceptive petrosal neurons of SH rats, we tested whether the sensitivity of these neurons to ATP differed between the rat strains. The application (15 nl) of ATP or α-β-methylene-ATP focally to the carotid body produced dose-dependent increases in the firing and membrane depolarization of petrosal neurons. Notably, petrosal neurons in the SH rat were more sensitive than those from Wistar rats; for ATP, EC_{50} (half-maximal effective concentration) values were 8.8 ± 2.2 versus 17.2 ± 1.3 in SH versus Wistar rats, respectively (P < 0.001; **Fig. 3c**,**d**), and for α-β-methylene-ATP, EC_{50} values were 9.4 ± 2.1 versus 16.2 ± 2.4 in SH versus Wistar rats, respectively (P < 0.05; **Supplementary Fig. 4a**,**b**). Consistent with these findings, the P2X3-receptor-sensitive inward current evoked by ATP in chemoreceptive petrosal neurons was greater in SH than in Wistar rats (P < 0.0001; **Fig. 3e**,**f**).

Systemic blockade of P2X3 receptors is antihypertensive

Next, we tested whether antagonism of carotid body P2X3 receptors lowers arterial blood pressure in SH rats. 1-h infusions of a highly selective, noncompetitive P2X3-receptor antagonist called AF-219 were made at 1, 4 and 8 mg/kg/h, i.v. This compound was preferred for use *in vivo* over the structurally related, more lipophilic antagonist AF-353 because AF-219 has been used clinically for other indications in humans[29] and does not cross the blood–brain barrier. We performed a pharmacokinetic analysis to assess plasma concentrations of AF-219 in SH rats (**Fig. 4a** and **Supplementary Fig. 5**), and subsequently found correlations between plasma drug concentrations, with falls in systolic blood pressure (SBP) (**Fig. 4a**). In SH rats, basal arterial pressure was 151 ± 3/109 ± 3 (SBP/DBP) mmHg. AF-219 produced a dose-dependent drop in arterial pressure (**Fig. 4a**); no response was observed in Wistar rats (**Fig. 4b**; **Supplementary Fig. 6**). At a dose of 8 mg/kg/h, SBP/DBP in SH rats fell by −28 ± 3/−26 ± 4 mmHg (**Fig. 4a**; n = 7, P < 0.001). Heart rate and respiratory frequency also decreased (by −38 ± 8 and −12 ± 3 beats and breaths/min, respectively; **Supplementary Fig. 6**; n = 7, P < 0.01). Notably, after carotid body resection in SH rats, a 30-min infusion of AF-219 (8 mg/kg/h) still caused a residual fall of −17 ± 3 mmHg in

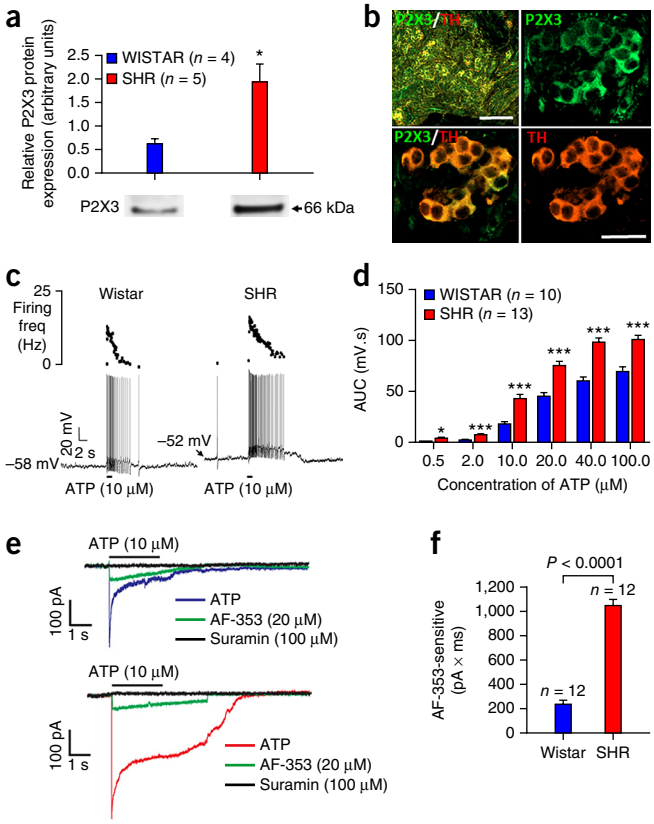

Figure 3 Upregulation of P2X3-receptor protein in the carotid body of SH rats and sensitization of chemoreceptive petrosal neurons to ATP in SH rats. (**a**) P2X3-receptor protein levels in the carotid body of Wistar and SH rats (Mann–Whitney *t*-test; $n = 4$ or 5). The relative expression of protein refers to the difference between rat strains. One pair of carotid bodies (left and right) from each rat was loaded per lane. (**b**) P2X3-receptor immunofluorescence (green) labeling on glomus cells identified by staining for tyrosine hydroxylase (red) (TH; $n = 3$ Wistar, $n = 3$ SH rats). The immunofluorescence images show a low (top left) and three high-power photomicrographs of carotid body glomus cells expressing TH and P2X3 receptors in a SH rat. See **Supplementary Figure 10** for control data on the specificity of the P2X3-receptor antibody. Scale bars, 100 μm (top left) and 25 μm (all others). (**c**) Whole-cell recording (current clamp) from petrosal chemoreceptive neurons in Wistar ($n = 10$) and SH rats ($n = 13$). ATP was micro-infused into the ipsilateral carotid body, as indicated. (**d**) The levels of membrane depolarization from petrosal chemoreceptive neurons evoked by increasing the concentrations of ATP applied to the carotid body in Wistar and SH rats. AUC, area under the curve. (**e**) Voltage-clamp recordings showing the inward current from identified petrosal chemoreceptive neurons evoked by the application of ATP to the ipsilateral carotid body in Wistar (top traces; $n = 12$) and SH rats (bottom traces; $n = 12$). The P2X3-receptor antagonist (AF-353, 20 μM) or the nonselective P2X-receptor antagonist suramin (100 μM) were applied to the ipsilateral carotid body before ATP, as indicated. (**f**) Quantification of the AF-353-sensitive, ATP-evoked inward current in Wistar and SH rats. Neurophysiological data were from the *in situ* arterially perfused preparation, and comparisons were made using an unpaired *t*-test. All data are means ± s.e.m. $^{*}P < 0.05$; $^{***}P < 0.001$.

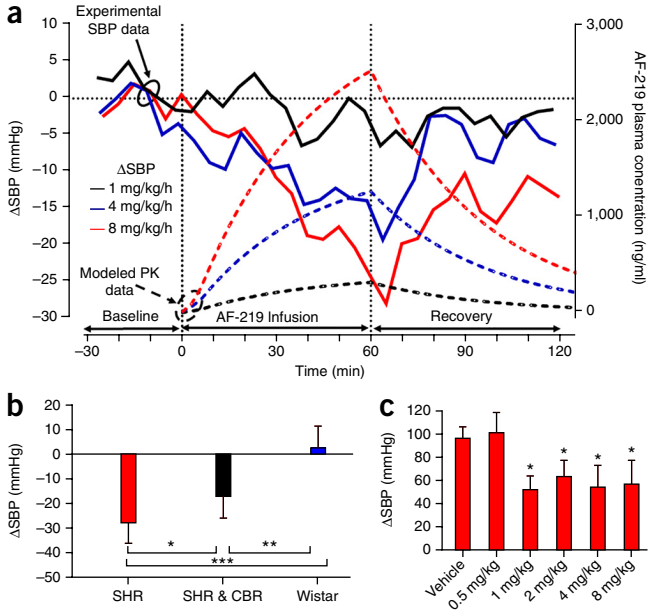

Figure 4 P2X3-receptor antagonism lowers arterial pressure in conscious SH rats. (**a**) Changes in SBP (solid lines) and the AF-219 plasma concentration predicted on the basis of plasma sampling (dotted lines; see also **Supplementary Fig. 5**) after intravenous infusion of the indicated doses of AF-219 (1, 4 and 8 mg/kg/h; $n = 7$ SH rats per drug dose). PK, pharmacokinetic. (**b**) Changes in SBP induced with 8 mg/kg/h AF-219 i.v. in SH rats without ($n = 7$) and with carotid body resection (SHR & CBR; $n = 7$) and in intact Wistar rats ($n = 4$). For CBR in SHR rats, measurements were performed before and after CBR in the same rat in four cases and in separate rats in three cases. (**c**) Peripheral chemoreflex-evoked pressor responses in SBP in conscious radiotelemetered SH rats during the infusion of vehicle or the indicated doses of AF-219 (0.5–8.0 mg/kg/h). $n = 7$ per group. Data are means ± s.e.m. One-way ANOVA Dunnett's post-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

SBP (**Fig. 4b**; $n = 7$, $P < 0.05$; **Supplementary Fig. 7**), which suggests P2X3-receptor-driven afferent activity of non-carotid body origin. The chemoreflex-evoked increase in arterial pressure in conscious SH rats was also reduced after systemic blockade of P2X3 receptors by AF-219 (bolus doses given i.v. >1 mg/kg; $n = 4$, $P < 0.05$; **Fig. 4c**). These data suggest that P2X3-receptor antagonism reduces aberrant afferent drive from the carotid body, thereby lowering blood pressure and normalizing its reflex sensitivity.

P2X3 receptor blockade reduces sympathetic tone in SH rats

To study the mechanisms by which P2X3-receptor blockade lowers arterial pressure in SH rats, we evaluated the effect of P2X3-receptor antagonism on cardiovascular autonomic activity. Ongoing basal levels of lumbar sympathetic nerve activity (SNA) recorded *in situ* were reduced from 20.8 ± 2.2 to 13.7 ± 2.5 μV (**Fig. 5a–c**; $n = 12$, $P < 0.001$) after focal delivery of AF-353 (15 nl, 20 μM) to carotid bodies bilaterally in SH rats. The SNA levels observed after AF-353 delivery were similar to those in normotensive rats either before (12.3 ± 2.6 μV) or after AF-353 application (11.7 ± 2.3 μV; **Fig. 5c**; $n = 12$, n.s.). Chemoreflex-evoked increases in expiratory-modulated SNA were 209 ± 12% and 131 ± 11% for SH and Wistar rats, respectively. After AF-353 delivery to carotid bodies, these levels were reduced significantly, to 76 ± 11% and 69 ± 7.8% in SH and Wistar rats, respectively (**Fig. 5a–e**; $n = 12$, $P < 0.001$); after AF-353 delivery, these levels were not different between rat strains. After 20–30 min, the depressant effects of AF-353 on SNA (both basal and reflex-evoked) were reversed (**Fig. 5b**) indicating that the effects of AF-353 on ongoing lumbar chain sympathetic activity chemoreflex-evoked discharge were reversible.

We replicated these data using 2′,3′-*O*-(2,4,6-trinitrophenyl) adenosine-5′-triphosphate tetra(triethylammonium) salt or TNP-ATP

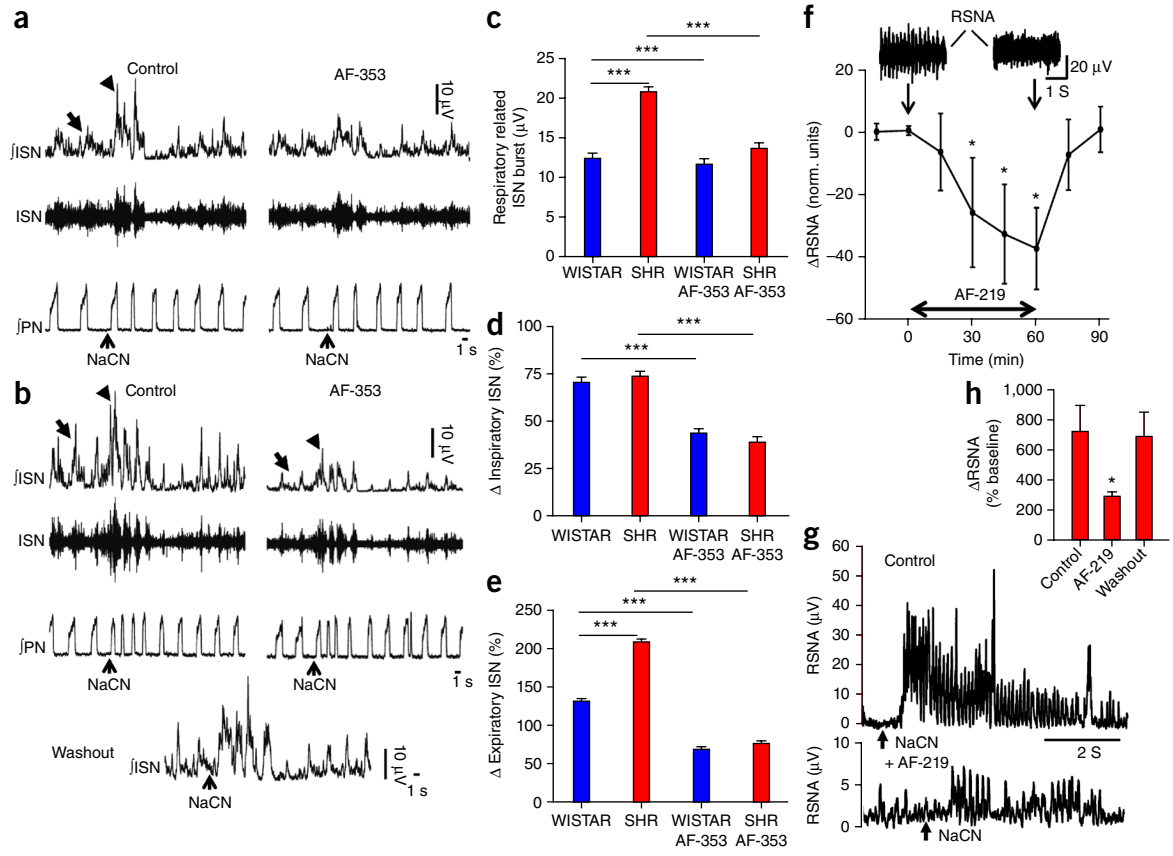

Figure 5 The antihypertensive action of P2X3-receptor antagonism is associated with a reduction in sympathetic activity in SH rats *in situ* and *in vivo*. (**a**,**b**) Ongoing lumbar chain sympathetic activity (arrow, raw: ISN; integrated: ∫ISN) and the sympathetic nerve reflex response (arrowhead) to peripheral chemoreceptor stimulation (evoked by NaCN, 22.5 µg i.a.) without (control) and with bilateral local application of AF-353 to the carotid bodies (20 µM, 15 nl) in Wistar (**a**) and SH rats (**b**) ($n = 12$ per group). Activity from the phrenic nerve (PN) (neural inspiration) was recorded, and its integrated form (∫PN) is shown. Also shown is a sympathetic nerve recording from the same preparation of SH rat after AF-353 washout. (**c**–**e**) Effects of AF-353 on basal respiratory modulated ISN (**c**), and chemoreflex-evoked changes in both inspiratory- (**d**) and expiratory- (**e**) modulated ISN in Wistar and SH rats ($n = 12$ per group). Inspiratory and expiratory modulation of ISN was determined by using averaged triggering from the integrated phrenic nerve (∫PN). One-way ANOVA Bonferroni post-test; data are means ± s.e.m. from *in situ* rat preparations. (**f**) Changes in renal sympathetic nerve activity (RSNA) recorded *in vivo* from conscious radiotelemetered SH rats ($n = 5$) during the infusion of AF-219 (8 mg/kg/h i.v.; vertical arrows indicate samples of raw RSNA and horizontal arrow indicates timing and duration of drug infusion). (**g**) A representative recording of the peripheral chemoreflex-evoked response (NaCN 22.5 µg i.a.) in the RSNA in a conscious radiotelemetered SH rat before (control) and after AF-219 infusion in the same animal. (**h**) Quantitation of mean data from **g**. In **h**, washout refers to the chemoreflex-evoked RSNA in SH rats the day after they had been infused with AF-219. Repeated measures one-way ANOVA, with Holm–Sidek *post hoc* comparison. $^{*}P < 0.05$, $^{***}P < 0.001$.

(**Supplementary Fig. 8**), a high-affinity, nonselective P2X-receptor antagonist that is structurally distinct from AF-353 and that inhibits P2X1, P2X3 and heteromeric P2X2/P2X3, with a 1,000-fold selectivity over homomeric P2X2, P2X4 and P2X7 receptors. This evidence supports the concept that the antihypertensive effects of AF-353 are due to the blockade of P2X3 homomeric and P2X2/P2X3 heteromeric receptors, rather than to nonselective actions on ionic conductances and/or other receptors.

Recordings were made from renal sympathetic nerves using radiotelemetry in conscious SH rats *in vivo* during chronic systemic P2X3-receptor antagonism using AF-219. This reduced both the basal renal sympathetic activity (−37 ± 13 normalized units relative to baseline; **Fig. 5f**; $P < 0.05$), which followed a similar time course to the fall in SBP (**Fig. 4a**), and the chemoreflex-evoked increase in renal sympathetic activity (**Fig. 5g**,**h**; $P < 0.05$). Washout data (**Fig. 5h**) indicated a reversal of the drug-evoked reduction in the chemoreflex-evoked renal sympathetic nerve response. Spontaneous renal sympathetic baroreflex gain was enhanced after AF-219 (0.14 ± 0.07 versus 0.01 ± 0.02 %/mmHg; $P < 0.05$), but not vehicle, infusion. Although no changes in autonomic activity to the heart were detected in conscious SH rats after AF-219 infusion (**Supplementary Fig. 9a**,**b**), spontaneous cardiac baroreflex was increased (0.13 ± 0.04 to 0.27 ± 0.05 ms/mmHg; **Supplementary Fig. 9c**; $P < 0.001$). We next tested the translatability of the SH rat data and potential use of a P2X3-receptor antagonist for human hypertension.

P2X3-receptor expression and tonic drive in human carotid bodies

If P2X3 receptors within the carotid body are to be considered as an antihypertensive target in humans, then an essential first step is a demonstration of their presence in human carotid bodies. Carotid bodies were obtained from cadavers of individuals with a medical history of hypertension, and P2X3 expression was determined by both immunocytochemistry ($n = 5$) and western blot ($n = 4$). P2X3-receptor antibody specificity was determined by using a blocking peptide (**Supplementary Fig. 10**). P2X3-receptor expression was prevalent in these carotid bodies (**Fig. 6a**,**b** and **Supplementary Fig. 11a**) and showed co-expression with the glomus cell marker tyrosine hydroxylase (**Fig. 6a**). Axons in the carotid body also expressed

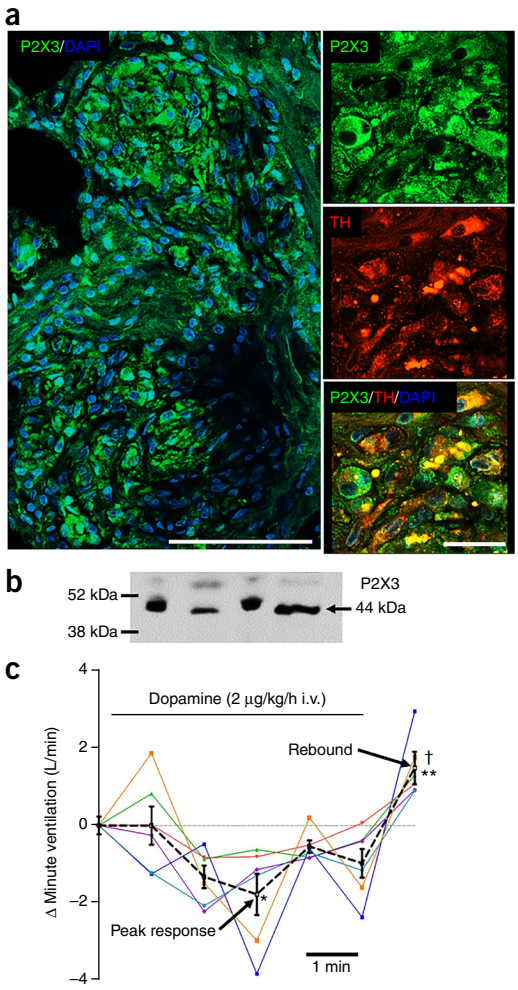

Figure 6 P2X3-receptor expression in carotid bodies from cadavers of individuals with a medical history of hypertension, and aberrant tone generation in carotid bodies from individuals with hypertension. (**a**) Left, a section of a carotid body from a human cadaver showing P2X3-receptor immunofluorescence (green) and nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI; blue; scale bar, 100 µm). Right, high-power confocal images showing co-localization of P2X3 receptor (green) and tyrosine hydroxylase (TH, red) (scale bar, 25 µm). Images are representative of those for five human carotid bodies. See **Supplementary Figure 10** for P2X3-receptor antibody control experiments and **Supplementary Figure 11** for axonal labeling. (**b**) Western blot of the P2X3-receptor protein in the human carotid body ($n = 4$). Arrow indicates bands at the molecular weight of the human P2X3 receptor (44 kDa). (**c**) Changes in minute ventilation during low-dose dopamine infusion (2 µg/kg/min i.v.) in six awake individuals with hypertension in the supine position (each solid line corresponds to one individual). A dextrose vehicle infusion was used as a control and reference at time zero. The mean (± s.e.m.) response is indicated by the black dotted line. Peak response was the lowest ventilatory response reached during the dopamine infusion. Note the appearance of a rebound hyperventilatory response after dopamine infusion was terminated. One-way ANOVA Bonferroni post-test. $^{*}P < 0.05$ vehicle versus peak depression; $^{**}P < 0.01$, depression versus rebound (mean of 5 min); $^{\dagger}P < 0.05$, vehicle versus rebound.

the P2X3 receptor (**Supplementary Fig. 11b**). This expression pattern was similar to that seen in SH rats (**Fig. 3b**).

Having verified the presence of P2X3 receptors in carotid bodies of humans with hypertension, we next tested whether tonicity in the carotid body of humans with hypertension exists, as assessed using low-dose dopamine infusion (5 min; 2 µg/kg/min i.v.), which was described previously, to inactivate the carotid body[38]. In six individuals with hypertension, we observed a biphasic response in minute ventilation to dopamine infusion (**Fig. 6c**), which comprised a depression during the infusion (−1.28 ± 0.52 l/min; $P < 0.05$), followed by an overshoot (relative to vehicle infusion) after the termination of dopamine infusion (1.28 ± 0.41 l/min; $P < 0.05$). These data indicate that the carotid body of humans with hypertension can generate tonic drive, and that dopamine infusion enables the identification of this aberrant discharge.

DISCUSSION

Many mechanisms that mediate reflex responses to hypoxia have been described in the carotid body that could contribute to its sensitization in disease states, including mechanisms involving two pore-domain potassium channels and acid-sensing ion channels[14,25], hydrogen sulfide[23,24], carbon monoxide[24], nitric oxide[24] and carotid body blood flow[27]. However, studies *in vitro* have also shown a major role for ATP release from glomus cells in response to hypoxia in the rat[30] and the cat[31], as well as in humans[35]. Whether there is a link between ATP release by these cells and any of the aforementioned mechanisms remains an open question. Moreover, P2X2- and P2X3-receptor subunits were both found to be present in the rat carotid body[36], and, by using co-cultures of glomus and petrosal cells *in vitro*, Nurse and colleagues[36] demonstrated that ATP acts via P2X receptors to excite petrosal neurons. This evidence was based on the use of suramin, a weak and nonselective P2X antagonist. P2X2-containing channels (P2X2 homomeric and/or P2X3 homomeric) probably dominate the normal physiological ventilatory response to hypoxemia, given that both P2X2 single-knockout and P2X2 and P2X3 double-knockout mice, but not P2X3 single-knockout mice, show ventilatory hyporeflexia[37]. Thus, changes in P2X3-subunit expression seem to be associated with pathological sensitization of carotid body reflexes.

In the present study, by using selective antagonists, we found that both exogenous and endogenous ATP released from the carotid body can act on P2X3-subunit-containing receptors on petrosal neurons. A limitation to our study is that we cannot fully establish the relative contribution of homomeric P2X3 versus heteromeric P2X2/P2X3 receptors in such responses. Although the antagonists that we used have a higher affinity for P2X3 homomeric than heteromeric P2X2/P2X3 receptors[28], it seems reasonable that P2X3 homomeric receptor upregulation in the carotid body could account for increased ATP sensitivity, because this form of the channel in recombinant studies displays substantially lower EC_{50} values than the homomeric receptor to the nucleotide[40]. In addition, the marked upregulation of *P2x3* mRNA rather than *P2x2* would probably precipitate a stoichiometric shift toward the expression of channels dominated by the P2X3 subunit. Data from voltage-clamp experiments indicate the presence of a nondesensitizing current to α-β-methyl-ATP recorded from chemoreceptive petrosal neurons and the probable involvement of P2X3 homomeric and P2X2/P2X3 heteromeric receptors.

Our data indicate that P2X3 receptors in the carotid body have a crucial role in controlling its reflex sensitivity in SH, but not in normotensive, rats. In SH rats, the upregulation of P2X3-receptor expression in the carotid body seems, in part, to be causal for the etiology of the tonicity, hyperreflexia of chemoreceptive petrosal neurons and downstream hyperactivity of the sympathetic nervous system. We acknowledge that chemoreceptive petrosal neurons could have a larger receptive field in the carotid body of SH, as compared to Wistar, rats, which could contribute to the hyperactive state of the petrosal

neurons. Notably, upregulated *P2x3* mRNA was exclusive to chemoreceptive petrosal neurons (i.e., it was not upregulated in nonchemoreceptive petrosal cells). The carotid body tonicity that we report from preparations *in situ* (1.5 Hz per petrosal neuron) most probably underestimates carotid body tonicity in conscious SH rats because the recordings were made under hyperoxic conditions, which are known to temper carotid body discharge[14,20]. The hyperoxic conditions might also explain the dearth of activity in neurons recorded from Wistar rats. Whether P2X3-receptor upregulation alone, without the need for increased ATP release or reduced ATP clearance, is sufficient for pathological carotid body signaling in the SH rat is unclear. However, given that the microvasculature of the carotid body is hypertrophied in the SH rat[41], and given the possibility that arterioles feeding the carotid body receive high sympathetic drive[41], the tissue is likely to be hypoxic and hypercapnic, a condition that is known to induce ATP release[42]. Additionally, inflammation, which is known to be present in the carotid body of SH rats[41], can also drive ATP release[43].

The association of P2X3-receptor upregulation and aberrant afferent signaling has been previously observed[28] and is consistent with the sensitization of visceral sensing mechanisms from, for example, the bladder[44,45], urinary tract[46], larynx[29], lungs[47], gastro-intestinal tract[48,49] and skeletal muscle[50] in numerous disease states, including chronic pain[51]. Elucidation of the mechanisms that control P2X3-receptor expression at both the transcriptional and cytosolic trafficking levels in the carotid body and other organs now becomes an important issue to enable the translation of P2X3 as a new drug target into the clinical arena.

The finding that P2X3-receptor activation evokes a rapidly inactivating current *in vitro*[52] seems inconsistent with an ability of P2X3 receptors to maintain tonic drive from the carotid body (or any organ). Notably, low pH, a postulated condition in the carotid body of SH rats[41], can potentiate P2X3-receptor inward currents and reduce desensitization[53]. Furthermore, the concept has been developed that P2X3-receptor activation might lead to local generator potentials and calcium transients in afferent terminals that lower the threshold for activation by any other excitatory agent, as described in somatosensory nociceptor C-fibers as leading to a condition of hyperalgesic priming[54,55]. Such threshold attenuation and chemoceptive priming might be under the control of the P2X3 receptor in the carotid body, although we do not rule out a possible involvement of P2X2/P2X3 heteromeric receptors, given that these channels show less rapid desensitization[52], which is consistent with our voltage-clamp data.

We found a robust dose-dependent lowering of arterial pressure after systemic administration of AF-219 to SH rats. AF-219 administration also led to a fall in blood pressure in rats with Goldblatt hypertension (W. Pijacka, F.M. McBryde & J.F.R. Paton, unpublished data), which indicates that the antihypertensive effect of P2X3 antagonism is not restricted to the SH rat. We propose that the effect of P2X3-receptor antagonism is primarily, but not exclusively, acting within the carotid body, given that the antagonist-induced fall in arterial pressure was reduced substantially, but not abolished, after carotid body ablation compared to intact rats. Further support for a direct action of AF-219 on the carotid body is that the fall in arterial pressure, which was accompanied by a reduction in sympathetic activity *in vivo*, was also observed after the direct injection of AF-353 into the carotid body *in situ*. The residual depressor response in the presence of AF-219 that remains after carotid body ablation suggests a contribution from other sensory afferents, in which P2X3 receptors have become active. Such sensory afferents could include glomus tissue in the thorax and abdomen, but this remains to be tested. Mechanistically, the decrease in sympathetic activity induced by P2X3-receptor antagonism included a reduction in its modulation by respiration, particularly by the expiratory component of respiration. It is this component that is upregulated in numerous models of hypertension[56,57], a finding that supports the importance of the central respiratory generator as a major driver of autonomic imbalance in hypertension[57]. P2X3-receptor antagonism also improved the spontaneous cardiac baroreceptor reflex, which possibly reflects the removal of the inhibitory influence of the carotid body on the carotid sinus baroreceptor reflex, as reported previously in both SH rats[20] and individuals with hypertension[58]. This improved baroreflex gain might contribute to the sympatho-inhibition and antihypertensive effects of P2X3-receptor antagonism in the SH rat.

The potential clinical importance of the upregulation of P2X3-receptor expression in the carotid body and petrosal neurons to pathology should not be underestimated. Administration of a P2X3-receptor antagonist might be able to attenuate aberrant signaling without affecting physiological function. Our data support this concept; carotid body tonic drive and hyperreflexia were abated by P2X3-receptor antagonism in hypertensive rats, yet the system remained physiologically responsive to stimulation, akin to control animals. These results are entirely consistent with the results of a recent human trial to treat chronic pathological cough, in which coughing was abated but physiological and protective cough reflexes did not seem to be altered by P2X3 receptor antagonism[29].

Peripheral chemoreception has both regulatory and protective-reflex functions[59]. Thus, an important consideration for the clinical use of a P2X3 antagonist such as AF-219 is whether it can abolish pathological signaling while preserving normal carotid body function. An issue for the clinical use of P2X3 antagonists might be the known adverse effects of P2X3 antagonism on taste perception[29], but appropriate dosing should be able to negate this.

Over the past 20 years, there has been a dearth of novel antihypertensive therapeutics[7]. We speculate that P2X3-receptor antagonism might be most efficacious in a subpopulation of humans with uncontrolled hypertension, demonstrable aberrant carotid body activity and an overactive sympathetic nervous system. The presence of P2X3 receptors in the carotid body of hypertensive humans, the release of ATP from human glomus cells in response to hypoxia[35] and the ability of dopamine infusion to select those individuals with aberrant carotid body discharge support the undertaking of a clinical trial to test the antihypertensive effects of P2X3 antagonism.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

W.P. conducted all *in vivo* radiotelemetry blood pressure studies and the rat and human immunocytochemistry and western blotting; this also included data analysis and figure and manuscript preparation. D.J.A.M. performed all *in situ* rat nerve and petrosal neuron whole-cell recording studies; this also included data analysis and figure preparation. M.P.d.S. performed the single-neuron PCR study. L.E.K.R. with A.K.N. carried out the diagnosis and recruitment of humans with hypertension, and L.E.K.R. with E.C.H. performed and analyzed data from the dopamine-infusion study. B.H.M. supported all *in situ* studies and assisted in experimental design, data analysis and manuscript preparation. F.D.M. conducted the *in vivo* radiotelemetry study for recording renal sympathetic nerve activity; this also included data analysis and figure preparation. A.P.A. performed some of the first immunohistochemistry on human carotid bodies. A.P.F. provided the P2X3-receptor antagonists, carried out the pharmacokinetic analysis and assisted in drug trial design and manuscript preparation and revision. J.F.R.P. orchestrated the design of the project, provided supervision with data acquisition and analysis, wrote the manuscript and revised it, and raised the funding.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Animal studies. All procedures conformed to the UK Animals (Scientific Procedures) Act 1986 and were approved by the University of Bristol ethical-review committee. We used male, spontaneously hypertensive (SH) or Wistar rats, which were bred within the animal facility of the University of Bristol. Animals were selected from different litters and housed with controlled temperature (21 ± 2 °C) and humidity (55 ± 10%). Wistar and SH rats were exposed to a 12 h day–night-shift cycle and had unlimited access to food and water. We used rats that were either 4–5 weeks old (70–100 g), for *in situ* preparations, or 14–16 weeks old, for radiotelemetry *in vivo*. Power calculations were based on: determining response magnitude (arbitrarily or on the basis of previous reports or pilot experiments), data variance (our previous experience, published papers and pilot experiments), taking into consideration the number of drugs to be tested and drug dosages, and the expected technical success rate of each type of experiment. Given the highly technical nature and the long duration of the studies, it was not possible to blind them.

Arterially perfused *in situ* juvenile rat preparation. Male Wistar or SH rats, 4–5 weeks, weighing 70–100 g were prepared as originally described[60]. In brief, rats were anesthetized deeply using isoflurane (5%). Anesthetic depth was assessed by a failure to respond to a noxious pinch of either a paw or the tail. Anesthetized rats were transected below the diaphragm, their upper bodies were submerged in ice-cooled Ringer solution and they were decerebrated pre-collicularly by gentle aspiration. The preparation was skinned and transferred to a recording chamber, and a double-lumen catheter was inserted into the descending aorta. One lumen was used to deliver perfusate pumped using a roller pump (Watson Marlow 505S). The perfusate was an isosmotic Ringer solution (containing in mM: NaCl 120, $NaHCO_3$ 24, KCl 5, $CaCl_2$ 2.5, $MgSO_4$ 1.25, KH_2PO_4 1.25, glucose 10) containing an oncotic agent (polyethylene glycol, 1.5%; Sigma-UK), gassed with carbogen (95% O_2 and 5% CO_2) and warmed to 32 °C, pH 7.3 after carbogenation and filtered using a nylon screen (pore size: 25-µm diameter). A side connector to this catheter enabled the administration of drugs directly into the arterial circulation. The second lumen of the catheter was used to monitor aortic perfusion pressure. The left phrenic nerve was isolated, and we recorded its activity (PNA) from the cut central end by using a glass suction bipolar electrode held in a 3D micromanipulator. Rhythmic ramping PNA gave a continuous physiological index of preparation viability. After respiratory-related movements commenced, a neuromuscular blocker (vecuronium bromide, 40 µg/ml, Norcuron Organon Teknika) was added to the perfusate to stabilize the preparation mechanically. Sympathetic nerve activity (SNA) was also recorded from the lumbar sympathetic chain by using a bipolar glass suction electrode. All nerve signals were AC-amplified, band-pass filtered (from 0.5 Hz to 5 kHz), rectified, integrated and sampled at 2–5 kHz. At the end of each experiment, the noise level was measured after the application of lidocaine (2%) to the sympathetic chain; this level was subtracted from the integrated signal. The head of the preparation was fixed by ear bars.

Whole-cell recordings from chemoreceptive neurons. The carotid body, carotid sinus nerve and petrosal ganglion complex was isolated on the animal's right side. By using recently described techniques[57,61], we performed whole-cell patch clamp recordings of chemoreceptive petrosal ganglionic or neurons from the nucleus tractus solitarii with electrodes filled with a solution containing the following (in mM): 130 K-gluconate, 4.5 $MgCl_2$; 14 trisphosphocreatine, 10 HEPES; 5 EGTA; 4 Na-ATP; 0.3 Na-GTP; pH 7.3. This solution had an osmolarity of ~300 mOsmol, and when filled, pipettes had a resistance of 3–8 MΩ when tested in bath solution. Current and voltage-clamp experiments were performed with an Axopatch-200B integrating amplifier (Molecular Devices) and pClamp acquisition software (version 10.0, Molecular Devices). Gigaseals (>1 GΩ) were formed, and whole-cell configuration was obtained by suction. To enable stable whole-cell recordings, the petrosal ganglion was opened along its lateral aspect. A mesh grid was lowered onto the ganglion for stabilization, while permitting visualization of the petrosal ganglia. We used electrical stimulation of the carotid sinus nerve (the axons of petrosal neurons) to find the chemosensitive petrosal and NTS neurons, as characterized by their excitatory response to sodium cyanide injected into the aorta (0.03%, 50 µl). ATP (15 nl of 0.5, 2, 10, 20, 40, 100 mM), α-β-methylene ATP (15 nl of 0.5, 2, 10, 20, 40, 100 mM) or AF-353 (15 nl of 20 µM; a P2X3-receptor antagonist)[40] was injected into the carotid body through the use of a 2-µm tip diameter glass microelectrode attached to a picopump (Picospritzer II, Parker Instrumentation).

Single petrosal neuron RT–qPCR. The cytoplasm of the chemosensitive petrosal and NTS neurons was pulled into a patch pipette with a slightly negative pressure, as described recently[61]. The cytoplasm was then placed into a microtube containing High Capacity cDNA Reverse Transcription Kit reagents (Life Technologies) and nuclease-free water for subsequent transcription in a thermocycler (Mastercycler Gradient, Eppendorf). A pre-amplification of the cDNA was performed using the TaqMan PreAmp Master Mix Kit (Life Technologies) with the following probes: Rn04219592_g1 (*P2x2*), Rn00579301_m1 (*P2x3*), TH: Rn00562500_m1, post-synaptic density-95: Rn00571479_m1 and β-actin: NM_031144.2 (reference gene). The pre-amplification protocol consisted of a hold temperature at 95 °C during 10 min and 14 cycles of 95 °C and 60 °C during 15 s and 14 min, respectively. The reactions for the single-cell RT–qPCR were performed in triplicate (StepOnePlus System, Applied Biosystems), using the same probes described above and the TaqMan Universal PCR Master Mix kit (Life Technologies), according to the manufacturer's recommendations. Water was used, instead of cDNA, as a negative control. β-actin was used as a control gene to normalize the data. The relative quantitation was determined by the $\Delta\Delta C_t$ method. For each sample, the threshold cycle (C_t) was determined and normalized to the average of the housekeeping genes ($\Delta C_t = C_{tUnknown} - C_{treferencegene}$). The fold change of mRNA content in the sample of petrosal neurons from SH rats relative to the Wistar group was determined by $2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t = \Delta C_{tUnknown} - \Delta C_{tControl}$. Data are presented as mRNA expression relative to that of the Wistar group. Dual probing of mRNA included TH and PSD-95 in 11 petrosal chemoreceptive cells from Wistar rats; TH and PSD-95 in nine petrosal chemoreceptive cells from SH rats; *P2x2* and *P2x3* receptor expression in six petrosal chemoreceptive cells from Wistar and five petrosal chemoreceptive cells from SH rats.

Blood pressure and renal nerve monitoring *in vivo*. Most surgical techniques were described previously[20]. Rats were anesthetized with ketamine (100 mg/ml; Vetalar, Zoetis, London, UK)/medetomidine hydrochloride (1 mg/ml Domitor, Elanco Animal Health, Hampshire, UK), injected intramuscularly.

For blood pressure recording only, an incision was made in the midline of the abdomen, and the abdominal aorta was exposed and cannulated just above the iliac bifurcation. The cannula of the transmitter (PA-C40; DSI, USA) was inserted until the tip rested just below the left renal artery branch point and was held in place with a tissue adhesive (VetBond, 3M, USA) and cellulose matrix. The transmitter body was placed in the abdominal cavity. Animals were allowed a 7-d recovery period. A nonsteroidal antiinflammatory analgesic was administered for 3–5 d postoperatively (0.006 mg/100 g of Metacam, Boehringer Ingelheim, Germany). Some rats were fitted with heparinized (TDMAC, Plolysciences, Eppelheim, Germany) catheters placed into the right jugular and left femoral vein for blood sampling and drug infusions, respectively. The catheter placed in the right jugular vein was perforated, according to a previous protocol[62]. Animals were allowed a 5-d recovery period after vein catheterization. Catheters were flushed with heparinized saline solution (0.9% saline/100 U heparin) every other day to maintain their patency.

For renal nerve recording, rats were implanted with telemetric devices (TRM56SP; Millar, Houston, Texas, USA). Details for blood pressure procedures are given above. For renal nerve recording, the right renal artery was exposed via a retroperitoneal approach, the renal nerves were gently freed from surrounding connective tissue, and a small piece of parafilm was placed underneath. The renal nerve(s) were then lifted over bipolar silver wire recording electrodes and isolated with a biocompatible silicone elastomer (Kiwk-Sil, WPI, Eu), and the nerve–electrode complex was secured in place with tissue adhesive and cellulose mesh. The incision was closed, and rats recovered for at least 7 d postoperatively, with analgesic treatment for 3 d (buprenorphine, 0.01 mg/kg per dose).

Experimental protocols for rat studies *in vivo*. These studies were repeated by two researchers who were working independently. Drug infusions (i.v.) were carried out in rats housed singularly. Animals were acclimated to the

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experimental environment for at least 2 h. At this time point, blood pressure remained stable. AF-219 was dissolved in 0.9% saline containing 10 mM HCl (vehicle). In some animals in which arterial blood pressure was measured, a pharmacokinetic analysis of AF-219 was performed by withdrawing blood from the jugular catheter at two time points: 1 h from the start of infusion and 1 h after infusion. Rats received an infusion of vehicle and 1, 4 and 8 mg/kg/h AF-219 for 60 min at 5 ml/kg/h into their femoral vein, followed by a 60-min washout period. The administration order of the drug doses and vehicle was randomized. These doses were selected by modeling pharmacokinetic data to obtain for rats a pharmacokinetic profile similar to that achievable for humans. The modeling was validated by quantifying AF-219 plasma concentrations after the 60-min infusion period by using high-pressure liquid chromatography assay and stable-labeled AF-219 as internal standard. The peripheral chemoreflex was tested with sodium cyanide (0.1 ml bolus i.v. 0.04% NaCN; BDH, Poole, UK, flushed with 0.9% saline (0.1 ml)). A bolus injection of sodium cyanide (0.04% in 0.1 ml) was given 45 min before, and 60 and 90 min after, the beginning of the infusion to check for chemoreflex sensitivity. Rats were allowed a 24-h recovery period between vehicle or drug and each drug dose. Infusions were carried out using a standard syringe pump (Harvard apparatus, MA, USA). Dopamine hydrochloride was infused (10 μg/kg/min; Sigma-Aldrich, Dorset, UK) to inactivate carotid bodies reversibly and assess their tonicity by measuring arterial pressure, heart rate and respiratory frequency.

Rats that had been tested with NaCN and dopamine hydrochloride underwent selective bilateral carotid body resection. We identified the carotid bifurcation via a midline incision through the skin on the ventral surface of the neck, and by using a binocular dissecting microscope. From a medial perspective, the carotid body was visualized and ablated using fine watchmakers forceps. Effective carotid body ablation was confirmed by an absence of a cardiovascular and respiratory response to NaCN (i.v.). Animals were allowed 6 d for recovery. Once selective carotid body ablation was confirmed, rats were infused with vehicle, AF-219 (8 mg/kg/h i.v.) and dopamine hydrochloride (10 μg/kg/min i.v.) on separate days.

Western blotting and immunocytochemical studies. Rat left and right carotid bifurcations were removed surgically from deeply anesthetized rats and immediately transferred into ice-cold saline. Carotid bodies were dissected under a Motic K Series Stereo Microscope (Ted Pella). Five SH and four Wistar rats were used for western blotting. The relative expression of protein refers to the difference between rat strains. One pair of carotid bodies (left and right) from each rat was loaded per lane. Six SH and six Wistar rats were used for immunohistochemistry. The specificity of the P2X3 receptor antibody was validated (**Supplementary Fig. 10**). Carotid artery bifurcations were removed, and carotid bodies were dissected from these and snap-frozen in liquid nitrogen (left and right together) for western blotting or fixed in ice-cold methanol/DMSO (4:1) for immunohistochemistry. Immunohistochemistry was carried out on the whole-mount rat carotid body and anti-P2X3-receptor antibody (APR026AN0202, Alomone, Israel). Incubation with anti-P2X3 and TH antibody was carried out in 2% goat serum overnight at 4 °C and gently mixed. All other experimental details were similar to those given below for the human studies.

Statistical analysis. Digitally transmitted arterial pressure and renal SNA signals were collected continuously during the drug or vehicle infusion period. Arterial pressure was measured during resting conditions. The renal SNA signal was amplified, filtered (50–5,000 Hz), full-wave rectified and integrated using a low-pass filter with a 20-ms time constant. Arterial pressure and renal SNA were sampled at 1k Hz using a 1401 acquisition system and purpose-written scripts in Spike2 software (Cambridge Electronic Designs, Cambridge, UK). From the arterial pressure, we derived pulse pressure, heart rate and respiratory frequency. Power spectral analysis was performed on heart rate using Spike2 software (CED instruments, Cambridge, UK). The interburst interval in renal SNA was taken as the baseline noise level and removed from the signal. The renal SNA signal was then scaled, with the 30 min immediately before infusion taken as the 100%-normalized level. We report the change in renal SNA with AF-219 relative to vehicle. The mean differences between baseline and treatment were analyzed with GraphPad Prism 6 (GraphPad Software, USA). We analyzed the spontaneous cardiac and renal sympathetic baroreflex gain (sBRG). The sBRG for the arterial pressure–renal SNA relationship was calculated for 10 min immediately before, and the final 10 min of the 60-min infusion period, of AF-219 or vehicle. DBP was smoothed over five beats, and positive or negative pressure ramps of at least five consecutive beats were identified automatically using Spike2 scripts (CED instruments, Cambridge, UK). All ramps in which both heart rate and renal SNA opposed the change in the corresponding DBP ramp were included for analysis. As described above, renal SNA was normalized with the baseline (pre-infusion) level set at 100%. A paired student *t*-test was used to compare the renal SNA sBRG between vehicle and AF-219 infusions.

The type of statistical test performed is indicated in the figure legends. Data were normally distributed and variance was comparable between the groups that were compared statistically. Unless stated in the figure legends, data were expressed as means ± s.e.m. and were assumed to be significant when $P < 0.05$. Source data for the Supplementary Figures is provided in **Supplementary Data Set 1**.

Drugs. Two highly potent and selective noncompetitive P2X3 (homomeric) and P2X2/P2X3 (heteromeric) receptor antagonists were used in these studies: AF-353 and AF-219 (Afferent Pharmaceuticals). Although the antagonists that we used have higher affinity for P2X3 homomeric receptors than for the heteromeric P2X2/P2X3 receptors[28,40,44], they cannot fully establish the relative contribution of homomeric, as compared to heteromeric, receptors. AF-353 was used in all studies *in situ*, which were performed before the *in vivo* studies, and at this time AF-353 was the only antagonist available to us. For subsequent studies *in vivo*, we used AF-219 because this drug is known not to cross the blood–brain barrier, whereas AF-353 does. The chemical formula for AF-219 is (amino-(4-isopropyl-2-methoxyphenyl)sulfone)-5-yl-(2,4-diaminopyrimidine-5yl)ether. The structure of AF-219 is contained in a published patent (see http://pdfaiw.uspto.gov/.aiw?PageNum=0&docid=20150057299&IDKey=DA73323D4481&). Doses used are based on IC_{50} (the concentration of the antagonist where the response is reduced by half) data[40,44]. For recombinant P2X3 homomeric receptors, the IC_{50} values for AF-353 (ref. 40) and AF-219 were ~8 nM and ~30 nM (unpublished data; A.P. Ford), respectively. Higher IC_{50} values were found for P2X2/P2X3 heteromeric receptors; for example, values were 100 nM and 250 nM for AF-353 and AF-219, respectively (unpublished data; A.P. Ford). Note that no inhibitory effect of either AF-353 or AF-219 on any non-P2X3 subunit containing receptors has previously been noted[40,44].

Human studies. All participants gave informed consent to participate in the studies, and consent was also obtained for removing carotid bodies from cadavers. All human studies were approved by the Central Bristol Research Ethics Committee (REC numbers: 14/SW/0054 and 12/SW/0277).

Carotid body tone in hypertensive humans. Small increases in circulating dopamine concentrations (≤2 μg/kg) can decrease peripheral chemoreceptor afferent input into the brainstem[63]. Thus, low-dose intravenous dopamine infusion (2 μg/kg/min) was used to test for any tonicity from carotid bodies. Six humans with hypertension (age 48.8 ± 3 years; BMI 28.6 ± 4, three female) with an office blood pressure of >140/90 mmHg (ambulatory blood pressure, >139/91 mmHg) and prescribed a mean of 2.83 different antihypertensive medicines were selected. Participants were given an intravenous infusion of dextrose (5%) for 5 min via peripheral venous access while breathing room air as a vehicle control. After the vehicle infusion, the line was purged and the intravenous infusion of dopamine (infusion concentration, 100 μg/ml of dopamine in 5% dextrose) was started for 5 min while breathing room air. Equivalent volumes of dextrose and dopamine were administered. Participants were blinded to the order of the infusions. This was followed by a recovery period of 5 min while breathing room air, allowing time for the dopamine to be metabolized. Respiratory volumes were measured via a respiratory flow-head connected to the expiratory port of a non-rebreathing valve attached to a face mask. Data were acquired using PowerLab and LabChart7 software (AD Instruments) and analyzed to calculate respiratory rate, tidal volume and minute ventilation using Spike2 (Cambridge Electronic Design) and MATLAB (MathWorks). The data was analyzed by GraphPad Prism 5 (GraphPad Software, USA).

Western blotting and immunocytochemical studies. Human left and right carotid bodies were obtained from twelve cadavers with a medical history of

hypertension (>140/90 mmHg). Briefly, carotid bifurcations were cut out, placed into cold ×1 PBS, and both carotid bodies were dissected and processed. The left carotid body was directly snap-frozen and used for western blotting, and the right carotid body was immediately fixed in 4% paraformaldehyde and used for immunohistochemistry.

Western blotting. The left human carotid body was crushed in liquid nitrogen with a mortar and pestle. Samples were subsequently homogenized with RIPA Lysis Buffer (Santa Cruz Biotechnology). Homogenate was centrifuged at 10,000*g* for 20 min at 4 °C, and the pellet was discarded. Protein concentration of the supernatant was determined by the Lowry method using the DC-protein assay kit (Bio-Rad Laboratories). Samples were mixed with loading dye (NuPage, LDS Sample Buffer 4×, Life Technologies), and 20 µg of protein was used for protein electrophoresis on 4–12% Bis-Tris gel (NuPage 4–12% Bis-Tris Gel, Life Technologies). The Amersham ECL Plex western blotting system using a low-fluorescent PVDF membrane (GE Healthcare, Buckinghamshire, UK) and Alexa Fluor 488 secondary antibody (Life Technologies, UK, cat. #R37116; diluted according to manufacturer's instructions) were used. This system enables detection and quantification with a broad dynamic range and high linearity. After protein transfer, the membrane was blocked for 1 h at room temperature with 2% Advance blocking agent (GE Healthcare). Membranes were incubated with anti-P2X3 (APR026AN0202; 1:200 dilution) overnight at 4 °C. The membranes were subsequently washed with 1 × PBS/0.1%Tween three times for 10 min at room temperature. Incubation with secondary antibody Alexa Fluor 488 was carried out in the dark at room temperature. Before imaging, the membrane was thoroughly washed. Signal was detected by scanning the membrane on a fluorescent laser scanner (Typhoon, GE Healthcare). Protein expressions were quantified using ImageQuant software (GE Healthcare). The specificity of the anti-P2X3 antibody was confirmed by membrane incubation with blocking peptide (APR026AG0140, Alomone; **Supplementary Fig. 10**).

Immunofluorescence. Human right carotid bodies were cut into 20-µm thick slices on a cryostat. Carotid body slices were incubated with 10% goat serum/0.1% Saponin (Sigma-Aldrich, UK) for 1 h at room temperature and gently agitated overnight. Primary antibody incubation with anti-P2X3 receptor (APR026AN0202, Alomone, Israel; 1:25 dilution) and anti–tyrosine hydroxylase (TH, F-11, sc-25269, Santa Cruz Biotechnology; dilution 1:25) was carried out, according to the manufacturer's recommendations, in 2% goat serum overnight at 4 °C. Samples were then washed in 1 × PBS four times for 10 min at room temperature, and the secondary antibody was applied for 1 h at room temperature. Anti-rabbit Alexa Fluor 488 was used to visualize the P2X3 receptor, and anti-mouse Alexa Fluor 594 was used to visualize tyrosine hydroxylase. Secondary-antibody incubation was followed by washing, as described above, and the samples were mounted on slides using mounting medium (Vectashield, H-1000, Vector Laboratories). Carotid bodies were examined under a Leica SP8 AOBS confocal laser-scanning microscope attached to a Leica DM I6000 inverted epifluorescence microscope, and imaging was performed using 'hybrid' GaAsP detectors for greater sensitivity. Images were processed in Adobe Illustrator CS3.

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